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(1390 REV. 5-93) US DEPT. OF COMMERCE PATENT &amp; TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER  
107976

**TRANSMITTAL LETTER TO THE  
UNITED STATES  
DESIGNATED/ELECTED OFFICE  
(DO/EO/US) CONCERNING A FILING  
UNDER 35 U.S.C. 371**

U.S. APPLICATION NO.  
(if known, sec 37 C.F.R.1.5)  
New U.S. National Stage of PCT/FR99/01247

**09/701243**

INTERNATIONAL APPLICATION NO.  
PCT/FR99/01247

INTERNATIONAL FILING DATE  
May 27, 1999

PRIORITY DATE CLAIMED  
May 27, 1998

## TITLE OF INVENTION

METHOD FOR AMPLIFYING AT LEAST ONE SPECIFIC NUCLEOTIDE SEQUENCE, AND PRIMERS USED

## APPLICANT(S) FOR DO/EO/US

Bruno MOUGIN and Ali LAAYOUN

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

**Items 11. to 16. below concern other document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ Entitlement to small entity status is hereby asserted.
16. ☒ Other items or information: translated PCT Request

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09/701243

JCO1 Rec'd PCT/PTO 27 NOV 2000

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) New U.S. National Stage of PCT/FR99/01247		INTERNATIONAL APPLICATION NO. PCT/FR99/01247		ATTORNEY'S DOCKET NUMBER 107976	
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17. <input checked="" type="checkbox"/> The following fees are submitted:  <b>Basic National fee (37 CFR 1.492(a)(1)-(5)):</b>  Search Report has been prepared by the EPO or JPO ....\$860.00  International preliminary examination fee paid to USPTO (37 CFR1.482) .....\$690.00  No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .....\$710.00  Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO .....\$1,000.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) .....\$ 100.00  <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				CALCULATIONS		PTO USE ONLY	

Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
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Claims	Number Filed	Number Extra	Rate		
Total Claims	12- 20 =		X \$ 18.00	\$	
Independent Claims	1- 3 =		X \$ 80.00	\$	
Multiple dependent claim(s)(if applicable)			+ \$270.00	\$	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$860.00	
Reduction by 1/2 for filing by small entity, if applicable.				-	\$
<b>SUBTOTAL =</b>				\$860.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 month from the earliest claimed priority date (37 CFR 1.492(f)).				+	\$
<b>TOTAL NATIONAL FEE =</b>				\$860.00	
				Amount to be refunded	\$
				Charged	\$

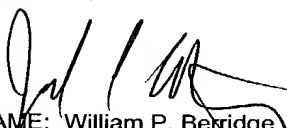
a. ☒ Check No. 114082 in the amount of \$860.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \$\_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Director is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 15-0461. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

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09/701243

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Bruno MOUGIN and Ali LAAYOUN

Application No.: New U.S. National Stage of PCT/FR99/01247

Filed: November 27, 2000

Docket No.: 107976

For: METHOD FOR AMPLIFYING AT LEAST ONE SPECIFIC NUCLEOTIDE  
SEQUENCE, AND PRIMERS USED

PRELIMINARY AMENDMENT

Director of the U.S. Patent and Trademark Office  
Washington, D. C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

IN THE CLAIMS:

Please cancel claims 1-12 without prejudice to or disclaimer of the subject matter  
contained therein.

Please add new claims 13-24 as follows:

## CLAIMS

- 13.. Method for amplifying at least one specific nucleotide sequence of a synthetic or natural nucleic acid contained in a reaction mixture, the reaction mixture consisting of at least one nucleic acid comprising at least two related nucleotide sequences and/or of at least two nucleic acids, each comprising at least one related nucleotide sequence; the method using at least one type of amplification primer capable of hybridizing with the nucleic acid so as to allow the amplification of the related nucleotide sequences consists in adding, to the reaction mixture, at least one sequence, acting as a blocking sequence, which is capable:
- of hybridizing to at least one nucleotide sequence, which is not the specific nucleotide sequence(s) to be amplified, and
  - of preventing, at the level of this nucleotide sequence, the elongation of the amplification primer.
14. Method according to claim 13, characterized in that the blocking sequence(s) is (are) capable of hybridizing to the, or to all the, nucleotide sequences which are not the specific nucleotide sequence(s) to be amplified.
15. Method according to claim 13, characterized in that each blocking sequence is an oligonucleotide based on modified nucleotides and/or ribonucleotides and/or deoxyribonucleotides, such as PNAs or thiophosphate nucleotides.
16. Method according to claim 13, characterized in that each blocking sequence comprises at least one element which prevents the amplification.
17. Method according to claim 16, characterized in that the element which prevents the amplification is located at the 3' end of the blocking sequence and does not allow its elongation.
18. Method according to claim 17, characterized in that the element which prevents the amplification is

located at the 5' end of the blocking sequence and acts as a protective element.

19. Method according to claim 16, characterized in that each element which prevents the amplification consists of a nucleotide or modified nucleotide, or of an oligonucleotide which may or may not comprise at least one modified nucleotide, the nucleotide, modified nucleotide or oligonucleotide not hybridizing to the nucleic acid.

20. Method according to claim 16, characterized in that each element which prevents the amplification consists of a molecule other than a nucleotide or than a modified nucleotide.

21. Method according to claim 16, characterized in that the element consists of at least five, in particular at least ten, and preferably at least fifteen, nucleotides or modified nucleotides or a mixture of nucleotide(s) and modified nucleotide(s).

22. Method according to claim 16, characterized in that the element is sufficiently long to allow the formation of a loop and of hybridization between the nucleotides and/or modified nucleotides which constitute this loop.

23. Method according to claim 16, characterized in that the element which does not allow the elongation is substituted for the hydrogen atom of the hydroxyl group or for the hydroxyl group, placed at the 3' position of the ribose, itself located at the 3' end of the nucleic acid.

24. Method according to claim 16, characterized in that the element is:

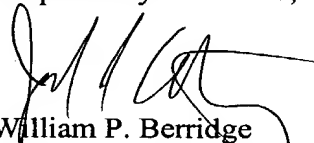
- substituted for the phosphate placed at in the 5' position of the ribose, itself located at the 5' end of the nucleic acid, or
- grafted onto the phosphate placed at the 5' position of the ribose, itself located at the 5' end of the nucleic acid.

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REMARKS

Claims 13-24 are pending. By this Preliminary Amendment, Claims 1-12 are canceled and Claims 13-24 are added.

Respectfully submitted,



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Date: November 27, 2000

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DEMANDE INTERNATIONALE PUBLIÉE EN VERTU DU TRAITE DE COOPERATION EN MATIÈRE DE BREVETS (PCT)

<b>(51) Classification internationale des brevets <sup>6</sup> :</b>  <b>C12Q 1/68</b>	<b>A1</b>	<b>(11) Numéro de publication internationale:</b> <b>WO 99/61661</b>  <b>(43) Date de publication internationale:</b> 2 décembre 1999 (02.12.99)
<b>(21) Numéro de la demande internationale:</b> PCT/FR99/01247  <b>(22) Date de dépôt international:</b> 27 mai 1999 (27.05.99)  <b>(30) Données relatives à la priorité:</b> 98/06866 27 mai 1998 (27.05.98) FR  <b>(71) Déposant (pour tous les Etats désignés sauf US):</b> BIO MERIEUX [FR/FR]; Chemin de l'Orme, F-69280 Marcy l'Etoile (FR).  <b>(72) Inventeurs; et</b> <b>(75) Inventeurs/Déposants (US seulement):</b> MOUGIN, Bruno [FR/FR]; 29, rue Lamartine, F-69003 Lyon (FR). LAAY-OUN, Ali [FR/FR]; Triangle Fleuri, 1, rue du Rhône, F-69007 Lyon (FR).  <b>(74) Mandataire:</b> CABINET GERMAIN & MAUREAU; Boîte postale 6153, F-69466 Lyon Cedex 06 (FR).		<b>(81) Etats désignés:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, brevet ARIPO (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), brevet eurasién (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), brevet européen (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), brevet OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Publiée</b> <i>Avec rapport de recherche internationale.</i>
<b>(54) Title:</b> <u>METHOD FOR AMPLIFYING AT LEAST A PARTICULAR NUCLEOTIDE SEQUENCE AND PRIMERS USED</u>		
<b>(54) Titre:</b> <u>PROCEDE D'AMPLIFICATION D'AU MOINS UNE SEQUENCE NUCLEOTIDIQUE PARTICULIERE ET AMORCES DE MISE EN OEUVRE</u>		
<b>(57) Abstract</b>		
<p>The invention concerns a method for amplifying at least a particular nucleotide sequence of a synthetic or natural nucleic acid contained in a reaction mixture, the reaction mixture consisting of at least a nucleic acid comprising at least two related nucleotide sequences and/or at least two nucleic acids each comprising at least a related nucleotide sequence; the method uses amplifying primers capable of hybridising with the nucleic acid for amplifying related nucleotide sequences. The invention also concerns primers for implementing such a method. Said method consists in adding, in the reaction mixture, at least a sequence acting as blocking primer capable of: hybridising with at least a nucleotide sequence, which is not the particular nucleotide sequence or sequences to be amplified; preventing at its level the elongation of the amplifying primer. The invention is particularly applicable in the field of techniques for amplifying related genes.</p>		
<b>(57) Abrégé</b>		
<p>La présente invention concerne un procédé d'amplification d'au moins une séquence nucléotidique particulière d'un acide nucléique de synthèse ou naturel contenu dans un mélange réactionnel, le mélange réactionnel étant constitué d'au moins un acide nucléique comportant au moins deux séquences nucléotidiques apparentées et/ou d'au moins deux acides nucléiques comportant chacun au moins une séquence nucléotidique apparentée; le procédé utilisant des amorces d'amplification capables de s'hybrider avec l'acide nucléique pour permettre l'amplification des séquences nucléotidiques apparentées. L'invention concerne également des amorces permettant la mise en oeuvre d'un tel procédé. Ce procédé consiste à ajouter, dans le mélange réactionnel, au moins une séquence, faisant office d'amorce de blocage, qui est capable: de s'hybrider à au moins une séquence nucléotidique, qui n'est pas la ou les séquences nucléotidiques particulières à amplifier, et d'empêcher à son niveau l'élongation de l'amorce d'amplification. L'invention trouve une application préférentielle dans le domaine des techniques d'amplification de gènes apparentés.</p>		

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Method for amplifying at least one specific nucleotide  
sequence, and primers used

5 The present invention relates to a novel method  
for amplifying at least one specific nucleotide  
sequence of a synthetic or natural nucleic acid  
contained in a reaction mixture. It also relates to  
primers which enable such an amplification.

10 The state of the art describes methods for  
amplifying nucleotide sequences using primers specific  
for these sequences to be amplified. Thus, it is  
possible to amplify a gene, or a family of genes, in a  
preparation of nucleic acids. Many techniques use  
oligonucleotides complementary to the target sequence  
15 which serve as primers for elongation by a polymerase.

For the amplification of DNAs, there exists the  
PCR (Polymerase Chain Reaction), as described in  
Patents US-A-4,683,195, US-A-4,683,202 and  
US-A-4,800,159, the LCR (Ligase Chain Reaction), set  
20 out for example in Patent Application EP-A-0,201,184,  
or the RCR (Repair Chain Reaction), described in Patent  
Application WO-A-90/01069.

For the amplification of RNAs, several  
techniques have also been described in various  
25 documents. These techniques are as follows:

- 3SR (Self Sustained Sequence Replication) with Patent  
Application WO-A-90/06995,
- NASBA (Nucleic Acid Sequence-Based Amplification)  
with Patent Application WO-A-91/02818,
- 30 - SPSR (Single Primer Sequence Replication) with Patent  
US-A-5,194,370, and
- TMA (Transcription Mediated Amplification) with  
Patent US-A-5,399,491.

35 However, these techniques impose a rigorous  
choice of amplification primers. Specifically, primers  
which are relatively nonspecific for the sequence of  
interest will allow the amplification of many related  
sequences to which they are bound. The amplicon  
corresponding to the sequence of interest will



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therefore be diluted in a mixture of amplicons, which will not facilitate the use of this amplification product. Under these conditions, it is then essential to rigorously choose a region of the nucleotide  
5 sequence which is sufficiently specific to allow a complementary primer which is just as specific to be produced. However, other problems may surface when choosing the sequence to be used for the hybridization of the primer. Specifically, the region which is really  
10 specific is sometimes unique and is located on the inner part of the sequence of interest. Choosing to hybridize a primer to such a region implies the production of only a varyingly significant fraction of said sequence of interest. There is therefore loss of  
15 information. In addition, the production of a primer specific for the nucleotide sequence of interest brings considerably more difficulty and work.

With the present invention, the risks of obtaining truncated amplification products and the  
20 difficulties in obtaining primers specific for the nucleotide sequence to be amplified are eliminated, since it is possible to amplify specifically the nucleotide sequence of interest under conventional hybridization conditions.

25 To do this, two types of complementary primer are used; firstly, a type of primer which hybridizes indifferently to all related nucleotide sequences and, secondly, a type of primer or [sic] each primer hybridizes to only one of these related sequences. The  
30 first, which are nonspecific, will be used as primers for the elongation, the second, which are specific for nucleotide sequences related to the sequence of interest, will be used as primers for blocking the elongation of some of these related nucleotide  
35 sequences.

When a mixture of nonspecific and specific primers is used, depending on the choice of the type of specific sequence used, the elongation of certain

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nonspecific sequences can be prevented. It is then possible to select the amplicons which are obtained.

Thus, the amplification of certain related sequences whose amplification is not desired can be  
5 blocked by adding complementary sequences specific for these related sequences, these specific complementary sequences acting as blocking primers. Thus, the sequence(s) of interest, which will be amplified selectively, is (are) isolated. A single amplicon is  
10 therefore obtained for each sequence of interest for which no blocking primer has been added.

To this effect, the present invention relates to a method for amplifying at least one specific nucleotide sequence of a synthetic or natural nucleic  
15 acid contained in a reaction mixture, the reaction mixture consisting of at least one nucleic acid comprising at least two related nucleotide sequences and/or of at least two nucleic acids, each comprising at least one related nucleotide sequence, the method  
20 using at least one type of amplification primer capable of hybridizing with the nucleic acid so as to allow the amplification of the related nucleotide sequences, characterized in that it consists in adding, to the reaction mixture, at least one sequence, acting as a  
25 blocking primer, which is capable:

- of hybridizing to at least one nucleotide sequence, which is not the specific nucleotide sequence(s) to be amplified, and
- of preventing, at the level of this nucleotide  
30 sequence, the elongation of the amplification primer.

Preferably, the blocking primer(s) is (are) capable of hybridizing to the, or to all the, nucleotide sequences which are not the specific nucleotide sequence(s) to be amplified.

35 Firstly, in the case of a blocking primer used in an amplification method, as described above, each blocking primer is an oligonucleotide based on modified nucleotides and/or ribonucleotides and/or deoxyribo-nucleotides, such as PNAs or thiophosphate nucleotides.

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Secondly, in the case of a blocking primer used in an amplification method, as described above, each blocking primer comprises at least one element which prevents the amplification.

5           Thus, the element which prevents the amplification is located at the 3' end of the blocking primer and does not allow its elongation.

          In addition, another element which prevents the amplification is located at the 5' end of the blocking  
10       primer and acts as a protective element.

          Each element which prevents the amplification consists:

- either of a nucleotide or modified nucleotide, or of an oligonucleotide which may or may not comprise at  
15       least one modified nucleotide, the nucleotide, modified nucleotide or oligonucleotide not hybridizing to the nucleic acid,
- or of a molecule other than a nucleotide or than a modified nucleotide.

20           In this case, the element consists of at least five, in particular at least ten, and preferably at least fifteen, nucleotides or modified nucleotides or a mixture of nucleotide(s) and modified nucleotide(s).

          According to a first embodiment, and when the  
25       element consists of a nucleotide or modified nucleotide, or of an oligonucleotide which may or may not comprise at least one modified nucleotide, the nucleotide, modified nucleotide or oligonucleotide does not hybridize to the nucleic acid and the element is  
30       sufficiently long to allow the formation of a loop and of hybridization between the nucleotides and/or modified nucleotides which constitute this loop.

          According to a second embodiment, and when the  
35       element consists of a nucleotide or modified nucleotide, or of an oligonucleotide which may or may not comprise at least one modified nucleotide, the nucleotide, modified nucleotide or oligonucleotide does not hybridize to the nucleic acid and the element consists of a "tail" of polynucleotides and/or of

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modified polynucleotides, which comprises all the same bases.

5 In the case of a blocking primer which is used in an amplification method, and which comprises an element which does not allow the elongation, the element is substituted for the hydrogen atom of the hydroxyl group or for the hydroxyl group, placed at the 3' position of the ribose, itself located at the 3' end of the nucleic acid.

10 In the case of a blocking primer which is used in an amplification method, and which also comprises a protective element, the element is:

- 15 - substituted for the phosphate placed at the 5' position of the ribose, itself located at the 5' end of the nucleic acid, or
- grafted onto the phosphate placed at the 5' position of the ribose, itself located at the 5' end of the nucleic acid.

20 The attached figures are given by way of explanatory example and are not limiting in nature.

Figure 1 represents a schematic view of the principle of an amplification of a strand of nucleic acid and of its complementary strand, using two primers; in this case, which is the simplest, there is one primer per strand.

Figure 2 represents a schematic view of the principle of an amplification according to Figure 1, but using the technique set out by the present invention.

30 Figure 3 represents the various substitutions which can be carried out on the nucleotides of the blocking primer, in which:

- 35 - R1 is an element which is at the 3' end of the blocking primer and which prevents any elongation during the amplification,
- R2 is an element which can be on at least one of the 2' positions of the ribose of a nucleotide of the blocking primer, and which reinforces the stability of the blocking primer/nucleic acid duplex, and

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- R3 is an element which is at the 5' end of the blocking primer and which acts as a protective element.

Figure 4 represents the positioning of the R1 element at the 3' end of the blocking primer when said  
5 primer is hybridized to the nucleic acid.

Figure 5 represents the positioning of the R3 element at the 5' end of the blocking primer when said primer is hybridized to the nucleic acid.

Figure 6 represents the blocking primer/nucleic  
10 acid duplex, in which X represents a nucleotide of the blocking primer comprising, in position on the ribose of this nucleotide, the R2 element which reinforces the stability of the duplex.

Figure 7 represents various structures which,  
15 by being added at the 3' position of the blocking primer, prevents [sic] any elongation during the amplification.

Figure 8 represents various structures which,  
by being added at the 5' position of the blocking  
20 primer, in addition to the modifications at the 3' position, as represented in Figure 3, act as a protective element by preventing the degradation or ejection of the blocking primer during the amplification.

Figure 9 represents the electrophoregram  
25 corresponding to the result of the sequence reaction for a sample of DNA from an HLA-DRB1\*1301 and HLA-DRB3\*01 lymphoblastoid line, observed for the region encoding amino acids 56 to 65 (according to HLA  
30 official nomenclature) of the HLA-DRB genes without using a blocking primer.

Figure 10 represents the electrophoregram  
corresponding to the result of the sequence reaction  
for a sample of DNA from an HLA-DRB1\*1301 and  
35 HLA-DRB3\*01 lymphoblastoid line, observed for the region encoding amino acids 56 to 65 (according to HLA official nomenclature) of the HLA-DRB genes using a 5'-phosphate/3'-C<sub>6</sub>-NH<sub>2</sub> oligonucleotide which inhibits the amplification of the HLA-DRB3 gene.

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Figure 11 represents the electrophoregram corresponding to the result of the sequence reaction for a sample of DNA from an HLA-DRB1\*0901 and HLA-DRB4\*01 lymphoblastoid line, observed for the region  
5 encoding amino acids 29 to 47 (according to HLA official nomenclature) of the HLA-DRB genes without using a blocking primer.

Figure 12 represents the electrophoregram corresponding to the result of the sequence reaction  
10 for a sample of DNA from an HLA-DRB1\*0901 and HLA-DRB4\*01 lymphoblastoid line, observed for the region encoding amino acids 29 to 47 (according to HLA official nomenclature) of the HLA-DRB genes using a 5'-acridine/3'-H oligonucleotide which inhibits the  
15 amplification of the HLA-DRB4 gene.

Figure 13 represents a schematic view of the principle of a selective amplification of a gene in a family of related genes located on the same chromosome.

The present invention therefore relates to,  
20 inter alia, the use of oligonucleotide primers modified at their ends, for selectively amplifying genes.

The invention also relates to a method using modified oligonucleotide primers, for selectively  
25 amplifying certain genes present in a set of related genes.

The analysis of genes of interest is facilitated by the use of gene amplification techniques which make it possible to prepare, from a biological sample, amounts of specific material which can be  
30 easily analyzed using the conventional techniques of molecular biology. Thus, the use of oligonucleotide primers bordering a gene region leads to the production of a mixture of nucleotide molecules which is considerably enriched in the molecule of interest,  
35 which molecule then becomes easily detectable using electrophoretic analysis techniques or molecular hybridization techniques. The effectiveness of this approach lies in the use of oligonucleotide primers specific for the gene regions to be analyzed. These

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primers must, therefore, be oligonucleotide sequences capable of hybridizing selectively with the nucleic acid sequences of interest which are present in the sample.

5           The analysis of genes which are members of families of structurally close genes can, however, sometimes be delicate. The search for nucleotide regions which are unique for a given gene makes it possible to achieve the desired specificity, but this  
10 approach can sometimes turn out to be difficult, or even impossible.

          The present invention consists, therefore, in combining the use of oligonucleotide primers specific for the amplification of a limited set of structurally  
15 close genes, and of modified oligonucleotide primers capable of specifically blocking the unwanted genes. In fact, each type of these modified primers corresponds to a single type of unwanted gene.

          This strategy makes it possible to simplify the  
20 analysis of genes in a mixture, by determining their nucleotide sequence (by sequencing in a gel, for example, or by multiple hybridization sequencing - DNA chip - ) or by analyzing mutations.

          The invention claims the use of mixtures of  
25 nucleotide primers enabling the effective amplification of corresponding nucleic acid regions, and of blocking nucleotide primers, which overlap or are located downstream (with respect to the nucleotide primer enabling the amplification), consisting of  
30 oligonucleotides which cannot serve as initiator sequences for the elongation, and therefore the amplification, of the downstream sequences.

          Thus, for an unwanted gene or allele, the nonblocking primer and the blocking primer hybridize to  
35 the same strand for a given primer polarity (5' primers upstream, or 3' primers downstream, of the region to be analyzed). The nonblocking primer, if it manages to hybridize to the strand to be amplified, cannot generate amplicons beyond the region corresponding to

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the hybridization site of the blocking primer, making the amplification corresponding to the nonblocking primer ineffective. This principle, illustrated in Figures 1, 2 and 13, concerns the functioning of the  
5 blocking primers and of the nonblocking primers.

According to Figure 1, an amplification is carried out with nonblocking primers P1 and P2. In an entirely conventional way, the extension of the P1 and P2 primers proceeds, and multiple amplicons A are  
10 obtained.

According to Figure 2, Figure 1 is repeated exactly, since it involves an amplification with nonblocking primers P1 and P2. However, on the complementary strand, and downstream of the progression  
15 of the P1 primer elongation, a sequence, acting as a blocking primer P1b, is added which is capable of hybridizing to the complementary strand and of preventing the amplification at the level of this strand. In this scenario, no amplicon will be produced.

According to Figure 13, Figures 1 and 2 are repeated exactly since it involves a selective amplification of the G<sub>2</sub> gene by blocking the related genes G1 [sic] and G3 [sic] using specific blocking primers and nonspecific amplification primers.  
20

The invention claims the use of blocking primers comprising modified nucleotides. This principle is illustrated in Figures 3 to 6.  
25

According to Figure 3, the nucleotide can be modified on the 2' or 3' positions of the ribose at the  
30 3' end of the oligonucleotide, and on the 5' position of the ribose at the 5' end of said oligonucleotide.

According to Figure 4, the R1 group replaces, at the 3' position of the ribose, the hydroxyl and makes it possible to prevent the elongation of the 3'  
35 end of the primer by the polymerase, when the nucleic acid/blocking primer duplex is formed.

According to Figure 5, the R3 group replaces, at the 5' position of the ribose, the phosphate and makes it possible to protect the blocking primer



against degradation of the 5' end and/or against being displaced, during the elongation of the amplification primer.

According to Figure 6, the nucleic acid/blocking primer duplex can be reinforced by substitution of the hydroxyl or of the hydrogen at the 2' position of the ribose, this substitution possibly taking place on several nucleotides of the blocking primer. The R2 group can be, for example, a 2'-O-methyl radical, which stabilizes the DNA/RNA duplexes by creating a hydrophobic-type interaction.

There are many uses for the claimed strategy each time a mixture of related sequences is to be analyzed: human or animal genetics, analyses of infectious agents (viruses, bacteria, parasites, etc.).

By way of example, uses in the domain of HLA (for Human Leukocyte Antigens) typing are described below.

Thus, the major histocompatibility complex (MHC) comprises a set of genes which are located on chromosome 6 in humans, and which are involved in regulating the immune response (Bodmer et al. *Nomenclature for factors of the HLA System*, 1996, Tissue Antigens, 1997, 49, 297-321). A very great polymorphism is observed for these genes, and an entirely specific set of versions (or alleles) of each of these genes is observed for each individual. It is important to note that any individual has two alleles of each gene, one inherited from the mother, the other from the father.

In this set of MHC genes, more commonly named "HLA genes", some are henceforth well known, both their nucleic acid sequence and the functions of the corresponding proteins. They are essentially HLA genes termed class I HLA genes (HLA-A, HLA-B, HLA-Cw) and HLA genes termed class II HLA genes (HLA-DR, HLA-DQ and HLA-DP). These genes participate in the regulation of the immune response at the level of the surveillance of the integrity of self, with various consequences in the

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medical domain. A first use relates to the field of organ or bone marrow grafts, and many studies have demonstrated the importance of optimum pairing, between the organ donor and the recipient, for the HLA genes  
5 therefore involved in the histocompatibility. A second use relates to the study of the susceptibility of each individual to develop certain pathologies induced by infectious agents (viruses, bacteria, parasites) or by other mechanisms which are still poorly understood (in  
10 the case of autoimmune diseases, for example). The HLA genes participate then in the development of the very great diversity of the immune response, observed in each individual, for a given ethnic group. Finally, the determination of the alleles of the HLA genes allows a  
15 precise characterization or identification of any individual, constituting a third domain of use of HLA typing.

One of the main difficulties of HLA typing lies in the structural homology observed for these genes,  
20 since they have evolved from common ancestors. It results therefrom that the genes of interest are to be analyzed within a set of close functional genes, or of nonfunctional genes (pseudogenes). It is therefore essential to control as well as possible the targeting  
25 of the analysis of the nucleic acid sequences, by optimizing the techniques for amplification of the regions to be sequenced.

For example, HLA-A typing is based on the selective analysis of the two alleles of the HLA-A gene observed in an individual, while avoiding the analysis  
30 of the structurally close genes HLA-B and HLA-Cw. It is therefore essential to be able to specifically amplify the related regions observed for the HLA-A gene, using nucleotide primers capable of hybridizing only with the  
35 regions targeted on this gene.

Another example relates to HLA-DR typing, in which the analysis of polymorphism concerns only the HLA-DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5 genes, which correspond to the genes encoding the polypeptide chains

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constituting the functional proteins expressed at the surface of the cells, while avoiding the co-amplification and analysis of the HLA-DRB2, HLA-DRB6, HLA-DRB7, HLA-DRB8 and HLA-DRB9 pseudogenes. The  
5 HLA-DR-typing example illustrates the great complexity of the mixture of nucleic acid sequences to be identified which are encountered for a given sample, and the presence of two alleles for each of the genes further increases the difficulty, sometimes making the  
10 interpretation of the results very delicate (typing ambiguities).

Taking HLA-DR typing as an example, it may prove that simplifying the analysis may be very beneficial, by restricting it only to the analysis of  
15 the HLA-DRB1 gene (with its two alleles for each individual), if the molecular analysis techniques employed use the determination of signal intensity (intensity of fluorescence for molecules of increasing sizes for sequencing) or the interpretation of  
20 oligoprobe hybridization reactivity profiles, for example. This objective may be attained by selecting nucleotide primers for HLA-DRB1-specific amplification, but this approach is not always possible because of the nucleic acid sequences observed for the various alleles  
25 of the HLA-DRB1 gene and of the sequences of the other HLA-DRB genes, which share great homology. One alternative consists of the present invention, and is based on the use of a mixture of primers which are specific for the HLA-DRB genes but nonspecific for the  
30 HLA-DRB1 gene, and of blocking primers which are specific for the HLA-DRB3, HLA-DRB4 and HLA-DRB5 genes. Selective amplification of the HLA-DRB1 genes therefore results therefrom, allowing the two HLA-DRB1 alleles observed for a given individual to be determined more  
35 easily.

The nucleotide primers are synthesized according to conventional methods, such as those using solid phase synthesis for example, and, unless otherwise stated, comprise an -OH residue in the 3'

region on the sugar (3'-OH), which allows their elongation during the amplification step. They are essentially oligonucleotides with a length of between 10- and 30-mer, according to the uses, this depending  
5 on the nucleic acid sequences considered.

The blocking nucleotide primers are prepared according to the methods mentioned above and contain a functional group, which inhibits the elongation, located at the 3' end of the oligonucleotide. The  
10 object of this blocking functional group is to prevent the addition, by the DNA polymerase, of the next base according to the information read on the complementary sequence. By way of example, this 3' blocking functional group may be a phosphate-alkylamine ( $C_6-NH_2$ ),  
15 phosphate or dabcyI group; with regard to this subject, see Figure 7. These groups protect the hydroxyl functional group (3'-OH), and thus block its reactivity during the polynucleotide polymerization catalyzed by the DNA polymerase. The blocking of the enzymatic  
20 polymerization can also be obtained by dehydroxylation of the 3' position. In fact, primers containing 3'-H, 2'-OH ends can be obtained using suitable reagents and the technique of oligonucleotide assembly on a solid support.

25 If the use of a blocking primer which does not overlap with the nonblocking primer must be envisaged, it may be advantageous to also protect the 5' end of the blocking primer, so that this primer is neither degraded by the exonuclease activity of the polymerase,  
30 nor displaced during the elongation of the nonblocking primer located further upstream (further to the 5' site) of the region to be amplified. For this, various modifications may make it possible to maintain the integrity of the blocking primer hybridized to the  
35 nucleic acid sequence to be inactivated. Several possibilities may be mentioned by way of examples: the acridine nucleus, dimethoxytrityl (DMT), the thiophosphate group and a "stem-loop" additional

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sequence. Such modifications are correctly represented in Figure 8.

The acridine nucleus is a powerful intercalating agent; it thus confers very great stability on the primer/target sequence duplex and avoids the displacement of the primer. The dimethoxytrityl (DMT) used as a protective group for the 5'-hydroxy end, the thiophosphate group used in the antisense strategy, and an additional sequence capable of forming a secondary "stem-loop" structure, protect the primer against possible degradation by exonuclease activity.

3' modification	-C <sub>6</sub> -NH <sub>2</sub> -phosphate -H -dabcyl
5' modification	-acridine -DMT -thiophosphate -"stem-loop" structure

Table 1: Modifications of the 3' (and) 5' ends of the blocking primers

The principle of using mixtures of nonblocking primers and of blocking primers can be used for the 3' end (downstream), or for the 3' end and for the 5' end (upstream of the region to be analyzed), depending on the characteristics of the nucleic acid sequences or depending on the complexity of the genes of the region to be analyzed.

This approach of amplification using blocking primers can also be used for strategies of simple amplification corresponding to a mixture of primers capable of hybridizing to the same nucleic acid sequence to be analyzed, or in multiplex corresponding to several mixtures of primers capable of hybridizing, during the same amplification reaction, to various

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nucleic acid sequences to be analyzed (various loci, various genes or various regions of a gene).

**Example of synthesis of 3'- and 5'-blocked oligonucleotide primers**

5           The blocked oligonucleotide primers were synthesized using an Expidite machine (Perseptive Biosystems) according to the automatic phosphoramidite method in accordance with the protocol proposed by the constructor. The phosphoramidite reagents required for  
10 the introduction of the 3' and 5' modifications were obtained from Glenn Research.

          The oligonucleotide purification is carried out by reverse phase HPLC (semipreparative 10 mm x 25 cm Beckman ODS column; C18; 5  $\mu$ m in porosity; eluent:  
15 30 min gradient of 10 to 30% of acetonitrile in a mixture with an aqueous solution of 0.1 M triethylammonium acetate at pH 7). The fractions containing the oligonucleotide were collected and dried. The various oligonucleotides were then taken up  
20 in pure water and quantified by measuring the UV absorption.

          To illustrate the principle of the present invention, two examples of use in the HLA domain are described below.

25 **Example 1: Specific blocking of the amplification of the HLA-DRB3 gene**

          Several HLA-DRB genes can encode an HLA-DR $\beta$  polypeptide chain: HLA-DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5. The organization of this set of functional  
30 genes transmitted in hereditary fashion varies according to individuals, who thus exhibit various conserved haplotypes. If the presence of an HLA-DRB1 gene is always observed, the presence of one or two other genes (HLA-DRB3, HLA-DRB4, HLA-DRB5) is  
35 facultative, depending on the HLA-DRB1 allele carried by the same chromosome. Moreover, because of the presence of the two haplotypes (one inherited from the mother, the other from the father), the complexity of the mixture of the HLA-DRB sequences to be analyzed is

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very variable. The analysis of the main information associated with the HLA-DRB1 alleles can therefore be difficult to interpret depending on whether the other HLA-DRB genes, such as for example HLA-DRB3, are present or not. It may therefore be advantageous to be able to amplify all the possible HLA-DRB1 alleles (184 listed in the 1997 nomenclature, *Nomenclature for factors of the HLA system, 1996, Tissue Antigens, 49, 3-II, March 1997*) without amplifying the HLA-DRB3 alleles possibly present (1 or 2 possible alleles among the 11 alleles listed in the 1997 nomenclature).

In order to illustrate the specific inhibition of the amplification of the DRB3 gene, two examples are described below, the first using an oligonucleotide comprising a 3' end modified by incorporating an amine-containing arm (oligonucleotide 5858, SEQ ID 3) and the second using an oligonucleotide comprising a modified 3' end comprising an -H, and a modified 5' end comprising an acridine (oligonucleotide 5967, SEQ ID 4).

The DNAs were extracted according to conventional techniques of cell lysis and of proteinase K digestion, and then purified by ethanol precipitation, after phenol extraction. The DNAs solutions (concentration adjusted to 100 ng/ $\mu$ l in H<sub>2</sub>O), are conserved at 2-8°C.

The general conditions used for amplification were as follows:

- 10X buffer	:	10 $\mu$ l
- Generic 5' primer (5867, SEQ ID NO 1) (10 $\mu$ M) (0.1 $\mu$ M final)	:	1 $\mu$ l
- Blocking 5' primer (10 $\mu$ M) (0 to 1.2 $\mu$ M final)	:	0 to 12 $\mu$ l
- Generic 3' primer (P2, SEQ ID NO 2) (10 $\mu$ M) (0.1 $\mu$ M final)	:	1 $\mu$ l
- dNTP (20 mM) (0.2 mM final)	:	1 $\mu$ l
- Taq polymerase (5 IU/ $\mu$ l) (1.5 U)	:	0.3 $\mu$ l
- DNA (100 ng/ $\mu$ l) (100 ng)	:	1 $\mu$ l
- H <sub>2</sub> O (QS)	:	100 $\mu$ l

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The characteristics of the amplification program used with a Perkin Elmer GeneAmp 9600 machine, were as follows:

- 5           2 min at 95°C (1 cycle)
- 30 sec at 95°C + 30 sec at 55°C + 30 sec at
- 72°C (32 cycles)
- 7 min at 72°C (1 cycle)

10           The amplification products obtained were controlled by analyzing an aliquot portion (5 µl) by agarose gel electrophoresis and then staining with ethidium bromide. After this control, the amplicons prepared were analyzed with the bioMérieux oligodetection HLA-DR-typing kit (Ref. 74 500). This

15           test makes it possible to determine the HLA-DR typing using a reverse hybridization technique in microplates, by detecting and analyzing the HLA-DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5 alleles (PCT/FR92/00702).

**DRB3 blocking with 5'-phosphate/3'-C<sub>6</sub>-NH<sub>2</sub> oligo:**

- 20   DNA: OMW line (ECCAC 9058), DRB1\*1301, DRB3\*01
- 5' generic HLA-DRB primer (oligonucleotide 5867, SEQ ID NO 1): 0.1 µM final
- 3' generic HLA-DRB primer (oligonucleotide P2, SEQ ID NO 2): 0.1 µM final
- 25   5' blocking HLA-DRB3 primer (oligonucleotide 5858, SEQ ID NO 3): 0, 0.3, 0.6, 0.9, 1.2 µM final

Microplate hybridization:

30           The values of the hybridization signals (optical density read at 492 nm x1000) observed for each of the specific probes are given in Table 2 below.



Final conc. of blocking primer 5858 ( $\mu$ M)	Probe 13	Probe 3+6	Probe 52a
0	> 2500	723	928
0.3	2109	578	101
0.6	1901	563	29
0.9	1759	374	31
1.2	1719	503	12

0.6/0 ratio	0.76	0.78	0.03
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Table 2: Signals for hybridization of the blocking primer 5858 to various target sequences as a function of its concentration

5

The calculation of the ratio of the value read for 0.6  $\mu$ M with respect to the value read without blocking, makes it possible to assess the inhibition of the amplification of the DRB3 gene.

10

Probes 13 and 3+6 are specific for the DRB1 gene, and probe 52a is specific for the DRB3 gene. The addition of oligonucleotide 5858 during amplification inhibits the amplification of the DRB3 gene without affecting the amplification of the DRB1 gene. This inhibition is dose dependent, and the total inhibition is observed for a blocking DRB3 oligonucleotide concentration of 0.6  $\mu$ M and above.

15

#### Sequencing:

The amplification products obtained with or without blocking of the DRB3 gene were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Ref. 4303152). The electrophoregrams of the Reverse reaction are given below for the assay without blocking and for the assay with blocking using 0.9  $\mu$ M of oligonucleotide 5858.

25

The montage of the electrophoregrams concerning the region encoding amino acids 56 to 65 (HLA official nomenclature) of the HLA-DRB genes illustrates the

inhibition of the amplification of the HLA-DRB3 gene (Figures 9 and 10).

Sequence expected for	5' > 3'
	56                      60                      65
DRB1*1301	CCT GAT GCC GAG TAC TGG AAC AGC CAG AAG GAC
DRB3*01	CCT GTC GCC GAG TCC TGG AAC AGC CAG AAG GAC
DRB1*1301+DRB3*01 (forward)	CCT GWY GCC GAG TMC TGG AAC AGC CAG AAG GAC
DRB1*1301+DRB3*01 (reverse)	GGA CWR CGG CTC AKG ACC TTG TCG GTC TTC CTG
Sequence read for	
assay without blocking	GGA CWR CGG CTC AKG ACC TTG TCG GTC TTC CTG
assay with blocking (0.9 µM)	GGA CTA CGG CTC ATG ACC TTG TCG GTC TTC CTG

5

Thus, the addition of DRB3-specific blocking primer (oligonucleotide 5858, 3'-C<sub>6</sub>-NH<sub>2</sub>) inhibits the amplification of the DRB3 gene, as shown by the disappearance of the related bases at positions 57 and 10 60, demonstrating the absence of the sequence corresponding to the DRB3\*01 allele.

**DRB3 blocking with 5'-acridine/3'-H oligonucleotide**

DNA: OMW line (ECCAC 9058), DRB1\*1301, DRB3\*01

15 5' generic HLA-DRB primer (oligonucleotide 5867, SEQ ID NO 1): 0.1 µM final

3' generic HLA-DRB primer (oligonucleotide P2, SEQ ID NO 2): 0.1 µM final

5' blocking HLA-DRB3 primer (oligonucleotide 5967, 20 SEQ ID NO 4): 0, 0.3, 0.6, 0.9, 1.2 µM final

Microplate hybridization:

The values of the hybridization signals (optical density read at 492 nm x1000) observed for each of the specific probes are given in Table 3 below.

- 20 -

Final conc. of blocking primer 5967 ( $\mu$ M)	Probe 13	Probe 3+6	Probe 52a
0	2356	907	893
0.3	1227	251	5
0.6	1395	239	0
0.9	799	185	4
1.2	965	161	0

0.6/0 ratio	0.60	0.30	0
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Table 3: Signals for hybridization of the blocking primer 5967 to various target sequences as a function of its concentration

5

The calculation of the "value read for 0.6  $\mu$ M"/"value read without blocking" ratio makes it possible to assess the inhibition of the amplification of the DRB3 gene.

10

Here again, the addition of oligonucleotide 5967 during amplification inhibits the amplification of the DRB3 gene without affecting the amplification of the DRB1 gene. This inhibition is dose dependent, and the total inhibition is observed for a blocking DRB3 oligonucleotide concentration of 0.3  $\mu$ M and above.

15

Example 2: Specific blocking of the amplification of the HLA-DRB4 gene

20

The situation is comparable to that described in Example 1. In order to simplify the interpretation of the HLA-DRB1 typing, it may be advantageous to limit the HLA-DRB amplification with the generic HLA-DRB primers, to the DRB1 gene, without co-amplification of the DRB4 gene. The present invention describes the use of DRB4-specific blocking primers.

25

The experimental protocols are identical to those described in Example 1.

DNA: T7526 line (ECCAC 9076), DRB1\*-0901, DRB4\*01

5' generic HLA-DRB primer (oligonucleotide 5867, SEQ ID NO 1): 0.1  $\mu$ M final

- 21 -

3' generic HLA-DRB primer (oligonucleotide P2, SEQ ID NO 2): 0.1  $\mu$ M final

5' blocking HLA-DRB4 primer (oligonucleotide 5965, SEQ ID NO 5): 0, 0.3, 0.6, 0.9, 1.2  $\mu$ M final

5 Microplate hybridization:

The values of the hybridization signals (optical density read at 492 nm x1000) observed for each of the specific probes are given in Table 4 below.

Final conc. of blocking primer 5965 ( $\mu$ M)	Probe 9	Probe 53
0	1769	1320
0.3	1935	110
0.6	1754	41
0.9	1750	29
1.2	1516	14

0.6/0 ratio	0.99	0.03
-------------	------	------

10 Table 4: Signals for hybridization of the blocking primer 5965 to various target sequences as a function of its concentration

15 The calculation of the "value read for 0.6  $\mu$ M"/"value read without blocking" ratio makes it possible to assess the inhibition of the amplification of the DRB4 gene.

20 Probe g is specific for the DRB1 gene, and probe 53 is specific for the DRB4 gene. The addition of oligonucleotide 5965 during amplification inhibits the amplification of the DRB4 gene without affecting the amplification of the DRB1 gene. This inhibition is dose dependent, and the total inhibition is observed for a blocking DRB4 oligonucleotide concentration of 0.6  $\mu$ M and above.

Sequencing:

The amplification products obtained with or without blocking of the DRB4 gene were sequenced using

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the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Ref. 4303152). The electrophoregrams of the Reverse reaction are given below for the assay without blocking and for the assay  
5 with blocking using 0.9  $\mu$ M of oligonucleotide 5965.

The montage of the electrophoregrams concerning the region encoding amino acids 29 to 47 (HLA official nomenclature) of the HLA-DRB genes illustrates the inhibition of the amplification of the HLA-DRB4 gene  
10 (Figures 11 and 12).

Sequence expected for	30	35	40	45
DRB1*0901	AGA GGC ATC TAT AAC CAA GAG GAG AAC GTG CGC TTC GAC AGC GAC GTG GGG GAG TAC			
DRB4*01	AGA TAC ATC TAT AAC CAA GAG GAG TAC GCG CGC TAC AAC AGT GAC CTG GGG GAG TAC			
DRB1*0901+DRB4*01 (forward)	AGA KRC ATC TAT AAC CAA GAG GAG WAC GYG CGC TWC RAC AGY GAC STG GGG GAG TAC			
DRB1*0901+DRB4*01 (reverse)	TCT MYG TAG ATA TTG GTT CTC CTC WTG CRC GCG AWG YTG TCR CTG SAC CCC CTC ATG			
Sequence read for				
assay without blocking	TCT MYG TAG ATA TTG GTT CTC CTC WTG CRC GCG AWG YTG TCR CTG SAC CCC CTC ATG			
assay with blocking (0.9 $\mu$ M)	TCT CCG TAG ATA TTG GTT CTC CTC TTG CAC GCG AAG CTG TCG CTG CAC CCC CTC ATG			

Thus, the addition of DRB4-specific blocking  
15 primer (oligonucleotide 5965, 3'-C<sub>6</sub>-NH<sub>2</sub>) inhibits the amplification of the DRB4 gene, as shown by the disappearance of the related bases at positions 30, 37, 38, 40, 41, 42 and 44, demonstrating the absence of the sequence corresponding to the DRB4\*01 allele.

20 The present invention can be used for high resolution HLA-DRB1 typing, the specific blocking of the specific amplification of the HLA-DRB3, -DRB4 and -DRB5 genes, and of the HLA-DRB2, -DRB6, -DRB7, -DRB8 and -DRB9 pseudogenes, using blocking primers reducing  
25 the analysis to a mixture of one (in the case of a homozygous sample) or of two (in the case of a heterozygous sample) nucleotide sequences. Such a strategy uses HLA-DRB3-specific blocking primers (oligonucleotides 5816 (SEQ ID NO 6), 5868  
30 (SEQ ID NO 7) and 5885 (SEQ ID NO 8), by way of examples), HLA-DRB4-specific primers (oligonucleotides 5883 (SEQ ID NO 9), 5916 (SEQ ID NO 10) and 5917 (SEQ ID NO 11), by way of examples) and HLA-DRB5-

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specific primers (oligonucleotides 5021 (SEQ ID NO 12), 5870 (SEQ ID NO 13), 5871 (SEQ ID NO 14), 5881 (SEQ ID NO 15), 5902 (SEQ ID NO 16), 5903 (SEQ ID NO 17), 5913 (SEQ ID NO 18) and 5914 (SEQ ID NO 19), by way of examples), modified at their 3' and 5' ends as described above.

Complete blocking of the HLA-DRB3, -DRB4 and -DRB5 genes can be produced using a mixture of blocking primers.

In Table 5 below, i represents inosine.

bioMérieux reference	SEQ ID NO	Nucleotide sequence (5' > 3')	5' Modif.	3' Modif.
5867	1	ATC CTT CGT GTC CCC ACA GCA CG	-	-
P2	2	TCG CCG CTG CAC TGT GAA G	-	-
5858	3	CCC CCC AGC ACG TTT CTT GGA GCT	-	C <sub>6</sub> -NH <sub>2</sub>
5967	4	CCC CCC AGC ACG TTT CTT GGA GCT	-acridine	-H
5965	5	CCC ACA GCA CGT TTC TTG GAG CAi GC	-	C <sub>6</sub> -NH <sub>2</sub>
5816	6	CCC AGC ACG TTT CTT GGA GCT	-	-
5868	7	CCC CCC AGC ACG TTT CTT GGA GCT	-	-
5885	8	CCC CCC AGC ACG TTT CTT GGA GiT	-	-
5883	9	CAT TTC CTC AAT GGG ACG GAG iiA	-	-
5916	10	CCC CCA GCA CGT TTC TTG GAG CAi GC	-	-
5917	11	CCC ACA GCA CGT TTC TTG GAG CAi GC	-	-
5021	12	CA CGT TTC TTG CAG CAG GA	-	-
5870	13	CA GCA CGT TTC TTG CAG CAG GA	-	-
5871	14	CA iGT TTC TTG CAG CAG GA	-	-
5881	15	CA GCA iGT TTC TTG CAG CAG GA	-	-
5902	16	CCC CCA GCA iGT TTC TTG CAG CAG GA	-	-
5903	17	CCC ACA GCA iGT TTC TTG CAG CAG GA	-	-
5913	18	CCC ACA GCA iGT TTC TTG CAG CAG iA	-	-
5914	19	CCC CCA GCA iGT TTC TTG CAG CAG iA	-	-

Table 5: Nucleotide sequence of the oligonucleotides  
used as primers for the amplification

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Inosine, which is not a natural base, is used in order to weaken the nucleic acid/blocking primer hybrid. Specifically, inosine is linked to its complementary nucleotide via two hydrogen bonds, and therefore, when it is substituted for a pyrimidine, the bonding between the two strands, in its region, is weaker. Since a gene can differ from other related genes by a single base, it is advantageous to substitute, on the blocking primer, which is complementary to the gene, the bases around this essential position with inosines. Since the nucleic acid/blocking primer duplex thus becomes weakened, hybridization can only occur if the primer is perfectly complementary to the target gene sequence. The specificity of the blocking primer is thus reinforced.

The present invention therefore relates to a method for selectively amplifying genes present in a mixture of related genes, using blocking oligonucleotide primers which correspond to oligonucleotides comprising a modified 3' end which does not allow their elongation during the steps for enzymatically amplifying target genes.

The invention also relates to the use of primers which block in the sense described in claim 1, and which comprise a modified 5' end which does not allow their displacement or their degradation, in the steps for enzymatically amplifying target genes using a primer specific for a region which is located further toward the 5' end on the same gene.

In the case of use of primers which block in the sense described above, the modification at the 5' end is facultative. Thus, two different possibilities exist.

According to a first embodiment, the 3' -OH group is replaced with a group which is not naturally found in nature, such as an -H, -phosphate, or -dabcyl group, or a carbon-based chain terminated by an -NH<sub>2</sub> group, by way of examples.





CLAIMS

1. Method for amplifying at least one specific nucleotide sequence of a synthetic or natural nucleic acid contained in a reaction mixture, the reaction mixture consisting of at least one nucleic acid comprising at least two related nucleotide sequences and/or of at least two nucleic acids, each comprising at least one related nucleotide sequence, the method using at least one type of amplification primer capable of hybridizing with the nucleic acid so as to allow the amplification of the related nucleotide sequences, characterized in that it consists in adding, to the reaction mixture, at least one sequence, acting as a blocking primer, which is capable:
  - of hybridizing to at least one nucleotide sequence, which is not the specific nucleotide sequence(s) to be amplified, and
  - of preventing, at the level of this nucleotide sequence, the elongation of the amplification primer.
2. Method according to claim 1, characterized in that the blocking primer(s) is (are) capable of hybridizing to the, or to all the, nucleotide sequences which are not the specific nucleotide sequence(s) to be amplified.
3. Blocking primer used in an amplification method according to either of claims 1 and 2, characterized in that each blocking primer is an oligonucleotide based on modified nucleotides and/or ribonucleotides and/or deoxyribonucleotides, such as PNAs or thiophosphate nucleotides.
4. Primer according to claim 3, characterized in that each blocking primer comprises at least one element which prevents the amplification.
5. Primer according to claim 4, characterized in that the element which prevents the amplification is located at the 3' end of the blocking primer and does not allow its elongation.
6. Primer according to claim 5, characterized in that the element which prevents the amplification is



- substituted for the phosphate placed at the 5' position of the ribose, itself located at the 5' end of the nucleic acid, or
  - grafted onto the phosphate placed at the 5' position
- 5 of the ribose, itself located at the 5' end of the nucleic acid.

**PCT****WORLD INTELLECTUAL PROPERTY ORGANIZATION****International Bureau****INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)**

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<b>(21) International application number:</b> PCT/FR99/01247  <b>(22) International filing date:</b> 27 May 1999 (27.05.99)  <b>(30) Data relating to the priority:</b> 98/06,866 27 May 1998 (27.05.98) FR  <b>(71) Applicant (for all designated States except US):</b> BIO MERIEUX [FR/FR]; Chemin de l'Orme, F-69280 Marcy l'Etoile (FR).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (US only):</b> MOUGIN, Bruno [FR/FR]; 29, rue Lamartine, F-69003 Lyon (FR). LAAYOUN Ali [FR/FR]; Triangle Fleuri, 1, rue du Rhône, F-69007 Lyon (FR).  <b>(74) Representative:</b> CABINET GERMAIN & MAUREAU; Boîte postale 6153, F-69466 Lyon Cedex 06 (FR).		<b>(81) Designated states:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> With the International Search Report.

As printed

(54) Title: METHOD FOR AMPLIFYING AT LEAST A PARTICULAR NUCLEOTIDE SEQUENCE AND PRIMERS USED

(54) Titre: PROCEDE D'AMPLIFICATION D'AU MOINS UNE SEQUENCE NUCLEOTIDIQUE PARTICULIERE ET AMORCES DE MISE EN OEUVRE

(57) Abstract

The invention concerns a method for amplifying at least a particular nucleotide sequence of a synthetic or natural nucleic acid contained in a reaction mixture, the reaction mixture consisting of at least a nucleic acid comprising at least two related nucleotide sequences and/or at least two nucleic acids each comprising at least a related nucleotide sequence; the method uses amplifying primers capable of hybridising with the nucleic acid for amplifying related nucleotide sequences. The invention also concerns primers for implementing such a method. Said method consists in adding, in the reaction mixture, at least a sequence acting as blocking primer capable of: hybridising with at least a nucleotide sequence, which is not the particular nucleotide sequence or sequences to be amplified; preventing at its level the elongation of the amplifying primer. The invention is particularly applicable in the field of techniques for amplifying related genes.

(57) Abrégé

La présente invention concerne un procédé d'amplification d'au moins une séquence nucléotidique particulière d'un acide nucléique de synthèse ou naturel contenu dans un mélange réactionnel, le mélange réactionnel étant constitué d'au moins un acide nucléique comportant au moins deux séquences nucléotidiques apparentées et/ou d'au moins deux acides nucléiques comportant chacun au moins une séquence nucléotidique apparentée; le procédé utilisant des amorces d'amplification capables de s'hybrider avec l'acide nucléique pour permettre l'amplification des séquences nucléotidiques apparentées. L'invention concerne également des amorces permettant la mise en oeuvre d'un tel procédé. Ce procédé consiste à ajouter, dans le mélange réactionnel, au moins une séquence, faisant office d'amorce de blocage, qui est capable: de s'hybrider à au moins une séquence nucléotidique, qui n'est pas la ou les séquences nucléotidiques particulières à amplifier, et d'empêcher à son niveau l'élongation de l'amorce d'amplification. L'invention trouve une application préférentielle dans le domaine des techniques d'amplification de gènes apparentés.

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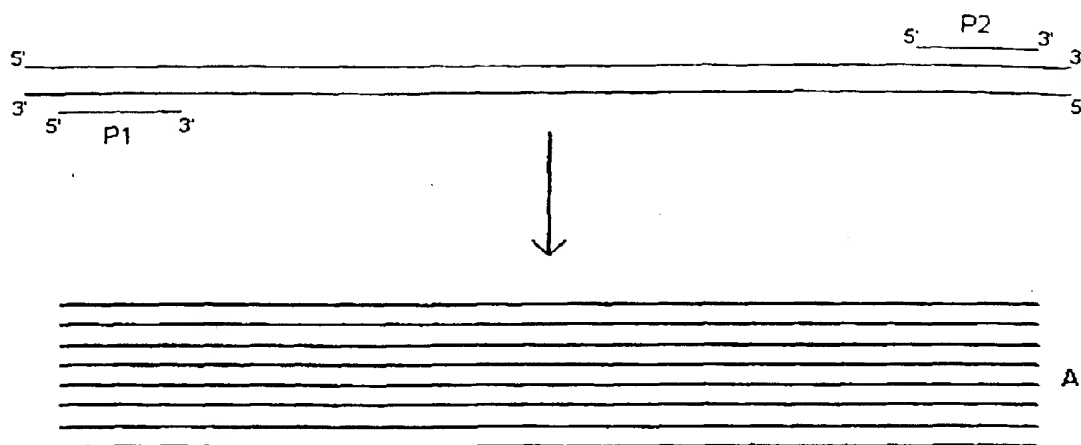
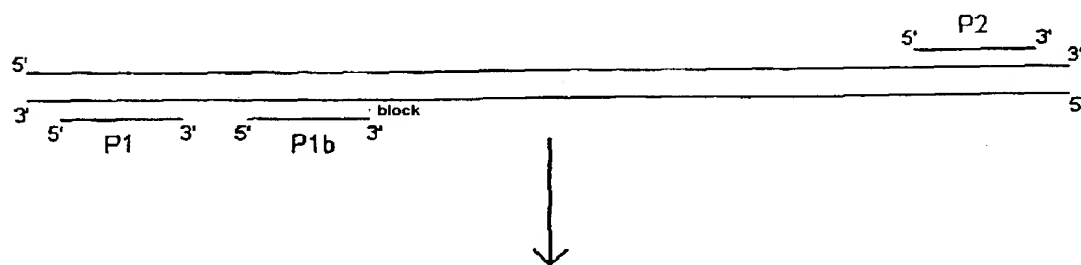
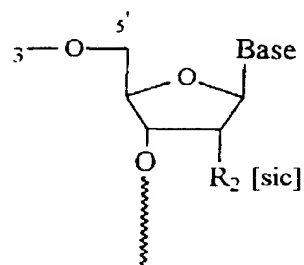
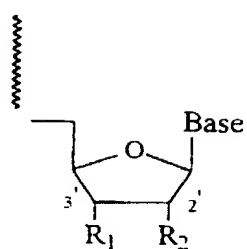


Fig. 1

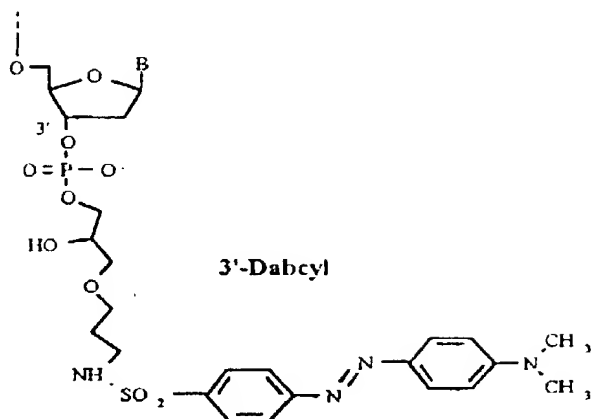
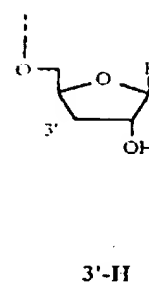
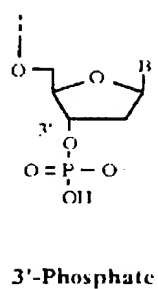
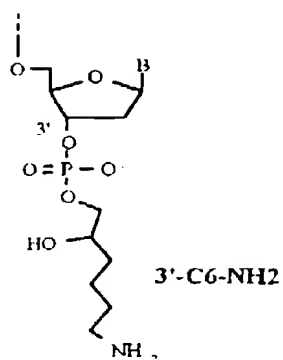


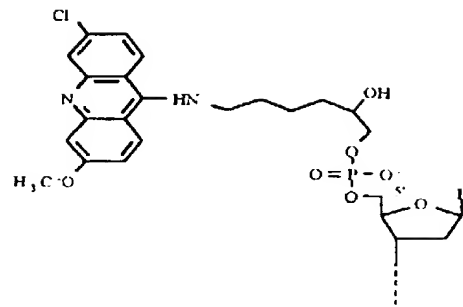
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Fig. 2

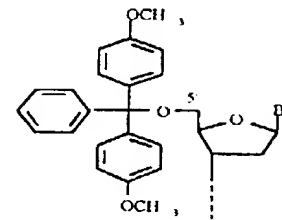
Fig. 3Fig. 4Fig. 5Fig. 6

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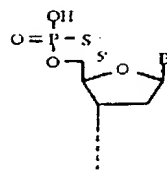
Fig. 7



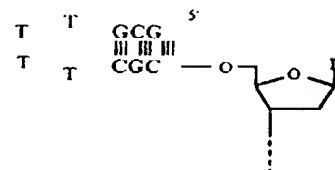
5'-Acridine



5'-DMT



5'-Thiophosphate



5'-stem-hoop

Fig. 8



3' 60 57 5'  
TCCITCTGGCTGTTCCAGKACTCGGCRWCAGG

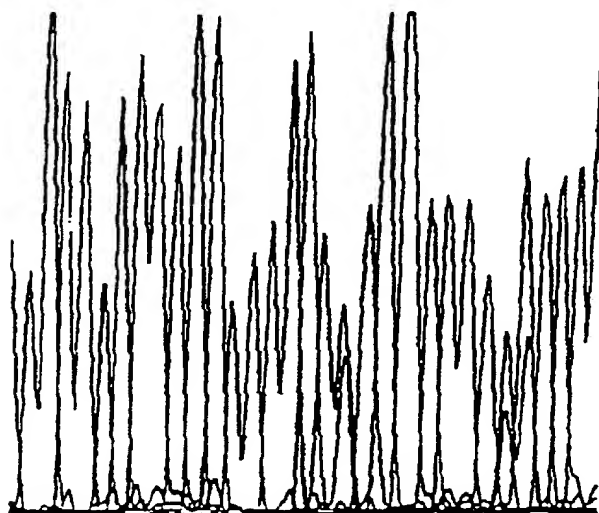


Fig. 9

3' 60 57 5'  
TCCTTCTGGCTGTTCCAGTACTCGGCATCAGG

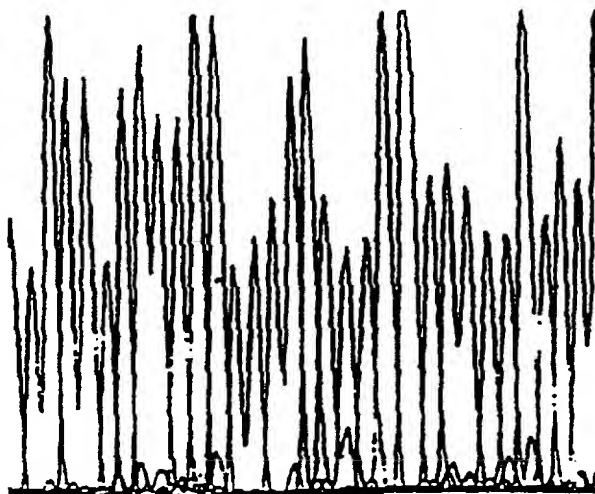
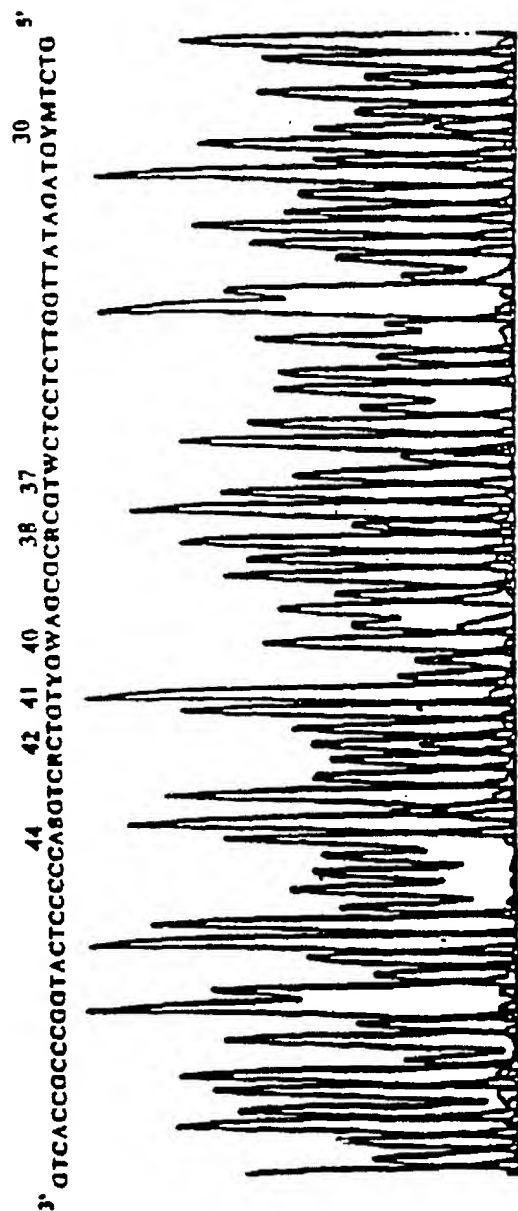
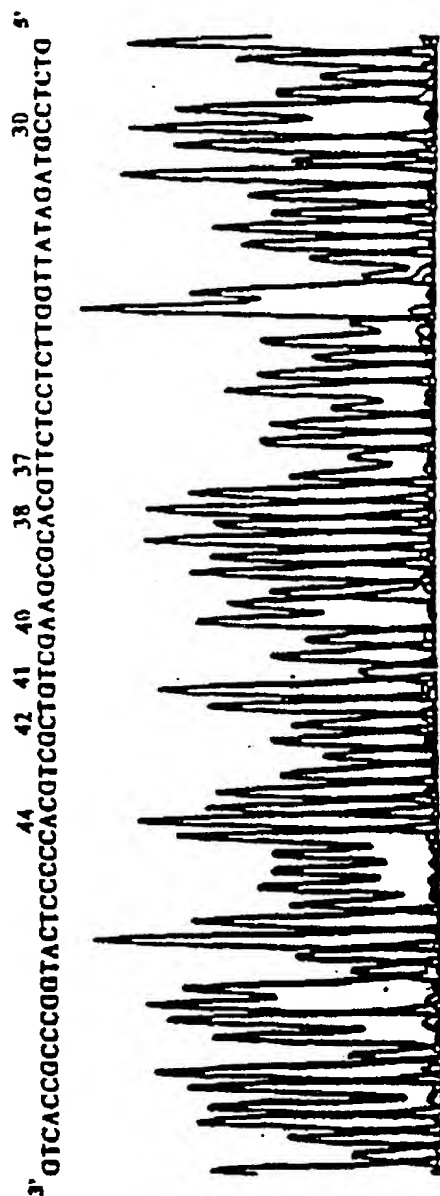


Fig. 10

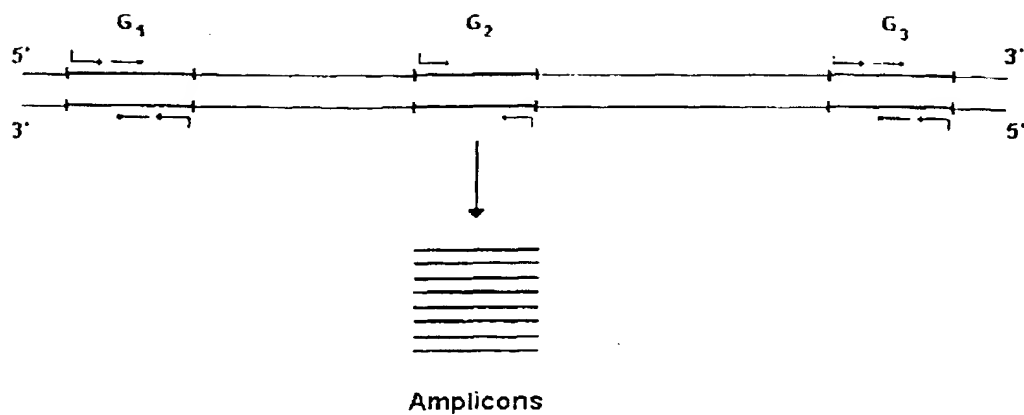
**Fig. 11**



**Fig. 12**



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→ Amplification Primer

← Blocking primer

Fig. 13

**DECLARATION AND POWER OF ATTORNEY  
UNDER 35 USC §371(c)(4) FOR  
PCT APPLICATION FOR UNITED STATES PATENT**

As a below named inventor, I hereby declare that:  
my residence, post office address and citizenship are as stated below under my name;

I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought, namely the invention entitled: Method for amplifying at least one specific nucleotide sequence, and primers used

described and claimed in international application number FR99/01247 filed 27th May 1999.

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

Under Title 35, U.S. Code §119, the priority benefits of the following foreign application(s) filed by me or my legal representatives or assigns within one year prior to my international application are hereby claimed:

French patent application No 98 06866 filed on 27 May 1998

The following application(s) for patent or inventor's certificate on this invention were filed in countries foreign to the United States of America either (a) more than one year prior to my international application, or (b) before the filing date of the above-named foreign priority application(s):

I hereby appoint the following as my attorneys of record with full power of substitution and revocation to prosecute this application and to transact all business in the Patent Office:

9 James A. Oliff, Reg. No. 27,075; William P. Berridge, Reg. No. 30,024;  
Kirk M. Hudson, Reg. No. 27,562; Thomas J. Pardini, Reg. No. 30,411;  
Edward P. Walker, Reg. No. 31,450; Robert A. Miller, Reg. No. 32,771;  
Mario A. Costantino, Reg. No. 33,565; Caroline D. Dennison, Reg. No. 34,494; and  
Stephen J. Roe, Reg. No. 34,463.

ALL CORRESPONDENCE IN CONNECTION WITH THIS APPLICATION SHOULD BE SENT TO OLIFF & BERRIDGE, PLC, P.O. BOX 19928, ALEXANDRIA, VIRGINIA 22320, TELEPHONE (703) 836-6400.

I hereby declare that I have reviewed and understand the contents of this Declaration, and that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1	Typewritten Full Name of Sole or First Inventor	<u>Bruno</u>	<u>MOUGIN</u>
2	Inventor's Signature	<u>Bruno</u>	<u>MOUGIN</u>
3	Date of Signature	<u>Nov 11 22</u>	<u>2000</u>
	Residence:	<u>LYON</u>	<u>France</u>
	Citizenship:	<u>French</u>	
	Post Office Address:	<u>29 Rue Lamartine 69003 LYON (France)</u>	
	(Insert complete mailing address, including country)		

Note to Inventor: Please sign name on line 2 exactly as it appears in line 1 and insert the actual date of signing on line 3.

IF THERE IS MORE THAN ONE INVENTOR USE PAGE 2 AND PLACE AN "X" HERE X

(Discard this page in a sole inventor application)

1 **Typewritten Full Name  
of Joint Inventor**2 **Inventor's Signature:**3 **Date of Signature:**

Residence:

Citizenship:

Post Office Address:

Ali

Given Name

Middle Initial

LAAYOUN

Family Name

November

Month

22

Day

2000

Year

LYON

City

State or Province

France

Country

Triangle Fleuri - 1 rue du Rhône 69007 LYON (France)

1 **Typewritten Full Name  
of Joint Inventor**2 **Inventor's Signature:**3 **Date of Signature:**

Residence:

Citizenship:

Post Office Address:

(Insert complete mailing  
address, including country)

Given Name

Middle Initial

Family Name

Month

Day

Year

City

State or Province

Country

1 **Typewritten Full Name  
of Joint Inventor**2 **Inventor's Signature:**3 **Date of Signature:**

Residence:

Citizenship:

Post Office Address:

(Insert complete mailing  
address, including country)

Given Name

Middle Initial

Family Name

Month

Day

Year

City

State or Province

Country

1 **Typewritten Full Name  
of Joint Inventor**2 **Inventor's Signature:**3 **Date of Signature:**

Residence:

Citizenship:

Post Office Address:

(Insert complete mailing  
address, including country)

Given Name

Middle Initial

Family Name

Month

Day

Year

City

State or Province

Country

Note to Inventor: Please sign name on line 2 exactly as it appears in line 1 and insert the actual date of signing on line 3.

This form may be executed only when attached to the first page of the Declaration and Power of Attorney of the application to which it pertains.

09701245 122500

09/701245

WO 99/61661

1

PCT/FR99/01247

JC01 Rec'd PCT/PTO 27 NOV 2000

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: BIOMERIEUX
- (B) STREET: CHEMIN DE L'ORME
- (C) CITY: MARCY L'ETOILE
- (E) COUNTRY: FRANCE
- (F) POSTAL CODE: 69280

- (ii) TITLE OF THE INVENTION: Method for amplifying at least one specific nucleotide sequence, and primers used

- (iii) NUMBER OF SEQUENCES: 19

(iv) CORRESPONDENCE ADDRESS:

- (A) NAME: Cabinet GERMAIN & MAUREAU
- (B) STREET: 12 rue Boileau
- (C) CITY: LYON
- (D) COUNTRY: FRANCE
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- (F) TELEPHONE: 04 72 69 84 30
- (G) TELEFAX: 04 72 69 84 31

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: DS, HD 3.5 inch disk
- (B) COMPUTER: EPSON (IBM compatible)
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: MICROSOFT WORD FOR WINDOWS

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear



- (ix) CHARACTERISTIC:
- (A) NAME/KEY: particular characteristic
  - (B) LOCATION: 1 (5' end)
  - (D) OTHER INFORMATION: modification by the acridine group

- (ix) CHARACTERISTIC:
- (A) NAME/KEY: particular characteristic
  - (B) LOCATION: 24 (3' end)
  - (D) OTHER INFORMATION: modification by H

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:  
CCCCCAGCA CGTTTCTTGG AGCT

24

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleotide
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) CHARACTERISTIC:
- (A) NAME/KEY: particular characteristic
  - (B) LOCATION: 24
  - (D) OTHER INFORMATION: G means inosine (i)

- (ix) CHARACTERISTIC:
- (A) NAME/KEY: particular characteristic
  - (B) LOCATION: 26 (3' end)
  - (D) OTHER INFORMATION: modification by the C<sub>6</sub>-NH<sub>2</sub> group

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:  
CCCACAGCAC GTTCTTGGA GCAGGC

26

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleotide
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear



4

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCCAGCACGT TTCTTGGAGC T

21

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CCCCCAGCA CGTTTCTTGG AGCT

24

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) CHARACTERISTIC:

(A) NAME/KEY: particular characteristic

(B) LOCATION: 23

(D) OTHER INFORMATION: G means inosine (i)

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCCCCAGCA CGTTTCTTGG AGGT

24

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) CHARACTERISTIC:

(A) NAME/KEY: particular characteristic  
 (B) LOCATION: 22  
 (D) OTHER INFORMATION: G means inosine (i)  
 (ix) CHARACTERISTIC:  
 (A) NAME/KEY: particular characteristic  
 (B) LOCATION: 23  
 (D) OTHER INFORMATION: G means inosine (i)  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:  
**CATTCCTCA ATGGGACGGA GGGA**

24

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 (A) LENGTH: 26 base pairs  
 (B) TYPE: nucleotide  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: cDNA  
 (ix) CHARACTERISTIC:  
 (A) NAME/KEY: particular characteristic  
 (B) LOCATION: 24  
 (D) OTHER INFORMATION: G means inosine (i)  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:  
**CCCCCAGCAC GTTCTTGGA GCAGGC**

26

(2) INFORMATION FOR SEQ ID NO: 11:  
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 (A) LENGTH: 26 base pairs  
 (B) TYPE: nucleotide  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: cDNA  
 (ix) CHARACTERISTIC:  
 (A) NAME/KEY: particular characteristic  
 (B) LOCATION: 24  
 (D) OTHER INFORMATION: G means inosine (i)  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

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26

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CACGTTTCTT GCAGCAGGA

19

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CAGCACGTTT CTTGCAGCAG GA

22

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) CHARACTERISTIC:

- (A) NAME/KEY: particular characteristic
- (B) LOCATION: 3
- (D) OTHER INFORMATION: G means inosine (i)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CAGGTTTCTT GCAGCAGGA

19

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## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) CHARACTERISTIC:

- (A) NAME/KEY: particular characteristic
- (B) LOCATION: 6
- (D) OTHER INFORMATION: G means inosine (i)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CAGCAGGTTT CTTGCAGCAG GA

22

## (2) INFORMATION FOR SEQ ID NO: 16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) CHARACTERISTIC:

- (A) NAME/KEY: particular characteristic
- (B) LOCATION: 10
- (D) OTHER INFORMATION: G means inosine (i)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCCCCAGCAG GTTCTTGCA GCAGGA

26

## (2) INFORMATION FOR SEQ ID NO: 17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) CHARACTERISTIC:

(A) NAME/KEY: particular characteristic

(B) LOCATION: 10

(D) OTHER INFORMATION: G means inosine (i)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCCACAGCAG GTTCTTGCA GCAGGA

26

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) CHARACTERISTIC:

(A) NAME/KEY: particular characteristic

(B) LOCATION: 10

(D) OTHER INFORMATION: G means inosine (i)

(ix) CHARACTERISTIC:

(A) NAME/KEY: particular characteristic

(B) LOCATION: 25

(D) OTHER INFORMATION: G means inosine (i)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CCCACAGCAG GTTCTTGCA GCAGGA

26

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) CHARACTERISTIC:

(A) NAME/KEY: particular characteristic

(B) LOCATION: 10

(D) OTHER INFORMATION: G means inosine (i)

(ix) CHARACTERISTIC:

